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DEVELOPMENT OF HIGH AMYLOSE WHEAT

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1 ABSTRACT

In a cereal grain starch accounts for two-thirds to three-quarters of the dry weight. Starch is made up of two glucan polymers, an essentially linear chain amylose and a highly branched molecule amylopectin. Wheat endosperm starch contains one-quarter amylose and the rest is amylopectin. Wheat grain starch with elevated amylose concentration (≅50%) is considered high amylose starch and has unique applications in food (resistant starch) and industrial uses. Therefore, high-amylose wheat starch can result in crop diversification and value-added processing in Saskatchewan. Amylose synthesis is complex, with starch synthases (SS) and starch branching enzyme (SBE) being the major enzymes which catalyse the formation of 1,4 and 1,6 glucosidic linkages, respectively. We have identified wheat lines with null alleles for SSII (A and B genomes) and SBEIIb (D genome) which were recombined to stack the null alleles. Some of the segregants had amylose concentration as high as 40%. These high amylose wheat lines are being used to introgress and stabilize the null alleles (SS and SBE) into adapted Canadian wheat cultivars to produce grain with high amylose starch.

2 EXECUTIVE SUMMARY

The main objective of this project was to increase the amylose concentration of wheat grain starch from 25% to more than 50%. Wheat grain with high amylose starch has unique applications, especially in food products where it can be used to produce resistant starch. Resistant starch is not digested in the human stomach thus releasing fewer calories than products with normal starch. However, the microbial flora in the large intestine breaks down the resistant starch and this has been suggested to have health benefits. The synthesis of amylose is complex and results from the interaction between starch branching enzymes and starch synthases. The objectives of this research were (i) to develop biochemical/molecular tools to identify wheat lines with null alleles for SBE and SS; and (ii) to develop wheat lines with high amylose concentration.

(i) Biochemical / Molecular tools to identify wheat lines with null alleles for SS and SBE:

(a) The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique has been modified to separate the SSII A, B and D genome products.

(b) A reverse transcription polymerase chain reaction (RT-PCR) technique using oligonucleotide primers based on the wheat SBE gene sequences has been optimized to differentiate between the *Sbe 2* null alleles from two genomes.

(c) Wheat lines with null SSII and SBEII null alleles have been identified and used in a breeding program.

(ii) Wheat segregants with 30 to 40% amylose have been developed.

At present the high amylose wheat lines are being stabilized and the null alleles are being stacked into adapted Canadian cultivars.

3 INTRODUCTION

Starch is the most abundant reserve carbohydrate in plants. In a cereal grain, starch accounts for two-thirds to three-quarters of the dry weight. Thus, much of the food consumed by humans is in the form of starch and it constitutes an excellent source of energy. Starch is stored as semicrystalline granules in the endosperm of a grain and is made up by two types of α-D-glucose polymers, amylose and amylopectin. Amylose (mol. wt. 250, 000 to 10^6) is a linear chain polymer of glucose units linked by α-1,4 linkages with a limited number (0.5%) of branches introduced by α-1,6 linkages. Amylopectin (mol. wt. 10^6 - 10^8) is a highly-branched α-D-glucose molecule with 4-5% of the glycosidic bonds being α-1,6 linkages. In normal cereal starches, amylopectin and amylose are present at a ratio of 3:1. Natural mutants with high amylose concentration have been reported for maize endosperm (Vineyard and Bear, 1952), pea cotyledons (Hilbert and McMasters, 1946), barley endosperm (Walker and Merritt, 1966) and rice endosperm (Satoh and Okuno, 1981). The high amylose mutants in maize and pea have amylose contents of 50% or higher as compared to 25-30% for the normal endosperm starch. The recently discovered high amylose mutants in japonica rice have 26 - 32% amylose as compared to 13 - 15% in the wild type. In mutant indica rice, the amylose content is 39 - 41%, to be compared to 24-25% for the wild type (Kaushik and Khush, 1991). The amylose fraction in the mutants has a lower molecular weight and wider size distribution than normal amylose. The amylopectin fractions of these starches are also more linear and have longer branch length than the normal starch.

The reduction in branch length and increased chain length is explained by the lack of one of the starch branching enzyme (SBE) isoforms. Starch branching enzyme introduces α-1,6 linkages in the glucose polymer to produce amylopectin. Two forms of starch branching enzymes, type I and type II are present in most cereals. In maize, the type II exists in two isoforms, IIa and IIb. All forms of branching enzymes share common properties but have slightly different substrate preference. Natural high amylose mutants in maize and rice are deficient in starch branching enzyme IIb (Boyer 1996). Elimination of wheat starch synthase II was recently reported to increase the amylose concentration in wheat starch (Yamamori et al., 2000).

4 OBJECTIVES

The overall objective of this project is to develop high amylose (≅ 50%) cultivars that are adapted to Saskatchewan. The specific objectives for the period 1997-2000 during the term of this project were:

1. To develop screening techniques to identify wheat lines carrying null alleles for starch branching enzymes (SBE) and starch synthase II (SSII).
2. To evaluate wheat germplasm for null *Sbe* and *SsII* alleles.
3. To produce wheat lines with null *Sbe* and/or *SsII* alleles.

5 MATERIALS AND METHODS

5.1 Seed Sources

Grains of various wheat (diploid, tetraploid and hexaploid) genotypes (1000 lines) were acquired from the USDA germplasm collection center at Aberdeen, Idaho, USA. Some of the *Triticum tauschii* lines from different geographical locations around the world were kindly provided by Dr. Bikram Gill, Wheat Genetics Resource Center, Kansas State University, Manhattan, Kansas, USA.

5.2 Donor Plants for Molecular/Biochemical Analyses:

To analyze starch granule protein profile and/or transcript analyses by reverse transcription polymerase chain reaction (RT-PCR), seeds were been planted in the soil and grown in a greenhouse, maintained at 25± 2°C day and 20± 2°C night under a 16h photoperiod (93 $\mu E/m^2$ per second) provided by banks of fluorescent tubes and incandescent lamps. . For RT-PCR analyses, plants were tagged at anthesis and spikes were harvested 10 – 15 dpa (days post anthesis) frozen immediately in liquid nitrogen and stored at -80°C until needed. For starch analyses plants were grown to maturity and seeds were collected and stored until ready for starch analyses

5.3 Plant Breeding Techniques: Standard techniques for wheat breeding were used to stack SSII and SBEIIb null alleles.

5.4 Isolation of Starch Granules

Starch granules were isolated from embryo-less half seed(s) according to the procedure described by Zhao and Sharp (1994, 1996) and Demeke et al., (1997, 1999). In a microfuge tube (1.5 mL) embryo-less single seeds were soaked in 1 mL of double distilled water and kept at 4°C overnight. After centrifugation at 15,000 x g for five minutes, the supernatant was removed and the pellet was crushed in 300 µL of double distilled water using a plastic pestle. The slurry was over-layered on 1 mL of 80% cesium chloride and centrifuged at 15,000 x g for 5 min. The starch granule pellet was washed twice with 1 mL of wash buffer (55 mM Tris-HCl, pH 6.8; 2.3% SDS, w/v; 5% ß-mercaptoethanol, v/v; and 10% glycerol, v/v), three times with water and once with acetone. The samples were centrifuged at 15,000 x g for three minutes after each washing step.

5.5 Extraction of Starch Granule Bound Proteins

Proteins from the starch granules were suspended in 100 µL of buffer (62.5 mM Tris-HCl, pH 6.8; 4% SDS, w/v; 5% ß-mercaptoethanol, v/v; and 10% glycerol, v/v), boiled for 5 min and then cooled on ice for 10 min. The cooled starch suspension was centrifuged for 20 min at 15,000 x g and the supernatant analyzed by gel-electrophoresis after the addition of 0.005% (w/v) bromophenol blue.

5.6 Electrophoretic (SDS-PAGE) Separation of Starch Granule Bound Proteins

For the analysis of SSII isoproteins, starch granule bound proteins were separated by discontinuous SDS-PAGE. The resolving gel contained 10% of 30:0.135% w/v acrylamide:bis-acrylamide; 375 mM Tris-HCl, pH 8.8; 0.1% SDS, w/v; 0.05% ammonium persulphate, w/v; and 0.05% TEMED, v/v. The stacking gel contained 126 mM Tris-HCl, pH 6.8; 4% of 30/0.8% acrylamide/ bisacrylamide, w/v; 0.1% SDS, w/v; 0.05% ammonium persulphate, w/v; and 0.05% TEMED, v/v. The running buffer contained 3.83 g Tris-base, 14.5 g glycine and 1.0 g SDS per liter of water (no pH adjustment). The gels (1.5 mm thick) were pre-run for 15 minutes at 100V, 50 µl of the starch granule bound protein supernatant was loaded into each well and the gels were run for 18-20 hr at 8 mA/gel. The separated proteins were visualized following staining of the gels using the Silver Stain Plus system (BioRad) according to the manufacturer's instructions.

5.7 Immunoblot Analyses of Starch Synthases:

Starch granule proteins separated by SDS-PAGE were transferred by vertical electroblotting (Sambrook et al., 1989) onto Immobilon nitrocellulose membranes (Millipore,Bedford, MA) at 1.4V/cm for 2.5 hr using buffer 3 described by Bolt and Mahoney (1997). The filters were blocked for 2hr in blocking buffer (5% [w/v] non-fat dry milk, 0.1% [v/v] Tween 20 in phosphate-buffered saline [Sambrook et al., 1989]) and subsequently incubated for 1hr with primary antibodies against SSII (Gao and Chibbar, 2000) in blocking buffer (1:6000 dilution). Blots were washed for 1 hr in blocking buffer, followed by incubation with alkaline phosphatase-conjugated goat-anti-rabbit secondary antibodies (Strategne) in blocking buffer (1:5,000 dilution). Thereafter, the membranes were washed with blocking buffer for 1hr and with 50 mM Tris-HCl, pH7.5, 150 mM sodium chloride for 45 min. Immuno-reactive polypeptides were revealed by

chemical staining with 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetrazolium (Stratagene) following the supplier's instructions.

5.8 RNA Isolation

RNA was isolated from developing endosperm using the previously described hot-phenol method (Båga et al., 1995). 100 mg of developing wheat kernels (7 or 14 days post-anthesis) were ground in a RNase free mortar and pestle that had been chilled with liquid nitrogen. The powder was transferred to a tube containing 0.8 ml acidic phenol that was pre-warmed to 65°C and the tube was vigorously shaken. One volume of RNA extraction buffer (65°C) was added (50 mM sodium acetate pH 4.5, 20 mMEDTA, 2% SDS, 50mM β-mercaptoethanol), the tubes were vigorously shaken, incubated for 5 minutes at 65°C, on ice for 5 minutes and then centrifuged for 5 minutes at 4°C and 15,000 x g. The aqueous phase (0.6 ml) was then added to 0.6 ml of acidic phenol (65°C), the tubes were vigorously shaken, incubated for 5 minutes at 65°C, on ice for 5 minutes and then centrifuged for 5 minutes at 4°C and 15,000 x g. RNA in the aqueous phase (0.5 ml) was precipitated by adding 1.4 ml of precipitation buffer (11mM sodium acetate pH 4.5, 95% ethanol), mixing by inversion, keeping on ice for 15 minutes then centrifuging the sample for 10 minutes at 15,000 x g and 4°C. The pellet was washed twice at room temperature with 1 ml 3M sodium acetate pH 5.2, once with ice-cold 80% ethanol, air dried and then resuspended in 50 µl of RNase free water. After quantitation of the RNA concentration using a spectrophotometer the samples were used immediately for reverse transcription or frozen at -70°C.

5.9 Reverse Transcription and PCR (RT-PCR)

One µg of total RNA was reverse transcribed using the SuperScript Choice System for cDNA synthesis (Gibco-BRL) according to the directions provided by the manufacturer. PCR, using SBEII specific forward (BE56) and reverse (BE59) primers, was then performed on the cDNA.

35 cycles of PCR was performed on the reaction mixture (25 µl final volume) that was comprised of 1X PCR Buffer (Boehringer-Mannheim: 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), dATP, dCTP, dGTP, dTTP (each 0.2 mM), 1 U Taq polymerase (Boehringer-Mannheim), 0.5 µl cDNA, the forward and reverse primers

(each 0.2 μM). The reaction products were separated on a 1% agarose gel containing 0.4 μg/ml ethidium bromide and then visualized under UV light.

5.10 Determination of Amylose Concentration

(a) *Megazyme Method*: Amylose concentration was determined according to the method of Gibson et al (1997) using the Megazyme Assay Kit (Megazyme International, Ireland). Wheat kernels were milled in a UDY cyclone mill (UDY Corporation, Fort Collins, CO, USA) and sifted through a 0.5 mm sieve. A 25 mg sample of the flour was boiled in 1 mL of dimethyl sulfoxide (DMSO) for 15 min. Ethanol (95%) was added to the boiled solution to precipitate and defat the starch. The defatted starch pellet was redissolved in 1 mL DMSO and diluted to 25 mL with 30% Con A solvent. Concentrated Con A solvent contained 0.52 M Sodium acetate, 3.0 M NaCl, 3.4 mM $CaCl_2$. 2H2O, 3.44 mM $MgCl_2.6H_2O$, and 3.54 mM $MnCl_2.4H_2O$, pH 6.4. A 0.5 mL aliquot taken from the 25 mL of solution was used to determine the total starch content by the amyloglucosidase/α-amylase method (Gibson et al 1997). Concanavalin A (Con A) was added to a 1.0 mL aliquot taken from the 25 mL of solution, to precipitate amylopectin. The amylose in the supernatant was hydrolyzed to glucose by amyloglucosidase/α-amylase, and the amylose concentration was expressed as a percentage of the total starch content.

(b) *HPLC Method*: To determine the amylose concentration by high performance size exclusion chromatography (HPSEC), a 5-mg starch sample was in 5 mL of double distilled-water in a glass tube and incubated at 130°C for 30 min. To 1 mL of the vortexed starch solution, 55 μL of 1M sodium acetate, pH 4.0, was added. The solution was vigorously mixed, and four units of isoamylase (200 units/mL stock solution, Megazyme) were added to debranch the starch. After 4 h of incubation at 40°C, the reaction mixture was boiled for 20 min to inactivate the isoamylase, and the starch solution suspended was freeze-dried. The debranched starch was dissolved in 200 μL of DMSO solution (99% DMSO and 1% nano pure water), and centrifuged at 15000 x g for 10 min. Forty microliters of the supernatant was injected into a PLgel 5 μM MiniMix-C guard column attached to a PLgel MiniMix 4.6-mm ID column (Polymer Laboratories, Inc., Amherst, Massachusetts, U.S.A.) to separate amylose and amylopectin using a high performance liquid chromatography system (Waters 600 Controller, Waters 610 Fluid

Unit, Waters 717 plus Autosampler, Waters 410 Differential Refractometer). The data were collected and analyzed using Millenium 2010™ Chromatography Software. Starch samples, column and detector were maintained at 40°C, 100°C and 45°C, respectively. Dimethyl Sulphoxide (99%) was used as an eluent at a flow rate of 0.2 mL/min. The amylose concentration of the starch samples was calculated by integration of the peak area corresponding to amylose, to that of the peak area corresponding to both amylose and amylopectin.

6 RESULTS AND DISCUSSION

Amylose synthesis is a very complex process in which various isoforms of starch synthases and starch branching enzymes catalyze the major steps. Both SBE and SSII are granule bound proteins, which can be visualized by SDS-PAGE (Fig. 1). The absence of SBEIIb has been associated with increased amylose concentration in maize, therefore our first strategy was to identify wheat lines deficient in SBEII. In wheat, both SBE I and II have very similar molecular mass, and therefore cannot be resolved by SDS-PAGE. Thus we used a RT-PCR strategy to screen the wheat germ plasm for null SBEII alleles.

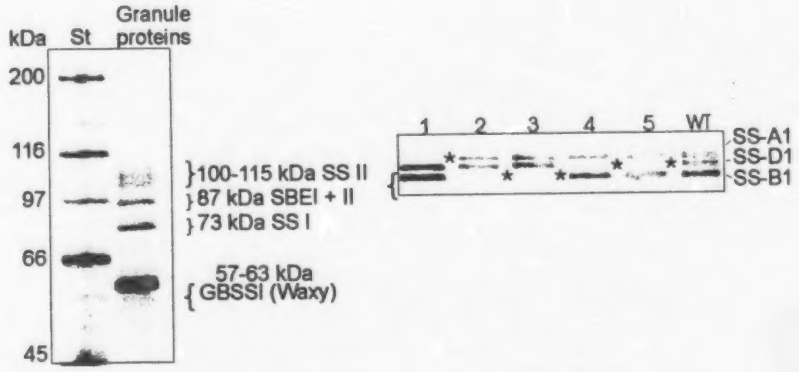

Figure 1: Separation of starch granule bound proteins by SDS-PAGE. The gel in the left panel shows the various starch granule proteins. In the right panel shows the separation of SSII from the three wheat genomes (A, B and D). Lane 1: null A, Lane 2: partial null B, Lane 3: null B, : Lane 4: partial null D, Lane 5: tetraploid wheat, WT : wild type.

6.1 Identification of null SBEIIb wheat lines:

RNA extracted from 12-15 dpa kernels from 450 wheat lines was screened for identifying Sbe2 null alleles. The primer pair BE 56 and BE 59 amplified two DNA products (Fig. 2) of approximately 350 and 400 bp. As shown in Fig 2, one line did not amplify the lower product. Based on our work with *T. tauschii* RNA we suggest that the missing product is from the D-genome. Therefore, this result suggests that we have identified a wheat line, with a null *Sbe2* allele. This wheat line was subsequently used in the breeding program. RT-PCR analyses of the progeny revealed that it inherited the null *Sbe2b* allele. Several other lines were also identified as being partial mutants for *Sbe*.

This was based on the reduced intensity of the Sbe RT-PCR amplification product stained with ethidium bromide. All the amplification products were extracted from gels and DNA sequenced to confirm their identity as *Sbe*.

Although we do have the *Sbe 1, 2* and *2b* genes isolated and characterized from wheat in our laboratory (Båga et al., 1999, 2000; Nair et al., 1997; Repellin et al., 1997; 2000), we were unable to identify more genome-specific oligonucleotide primers for use in RT-PCR to identify null *Sbe2b* alleles.

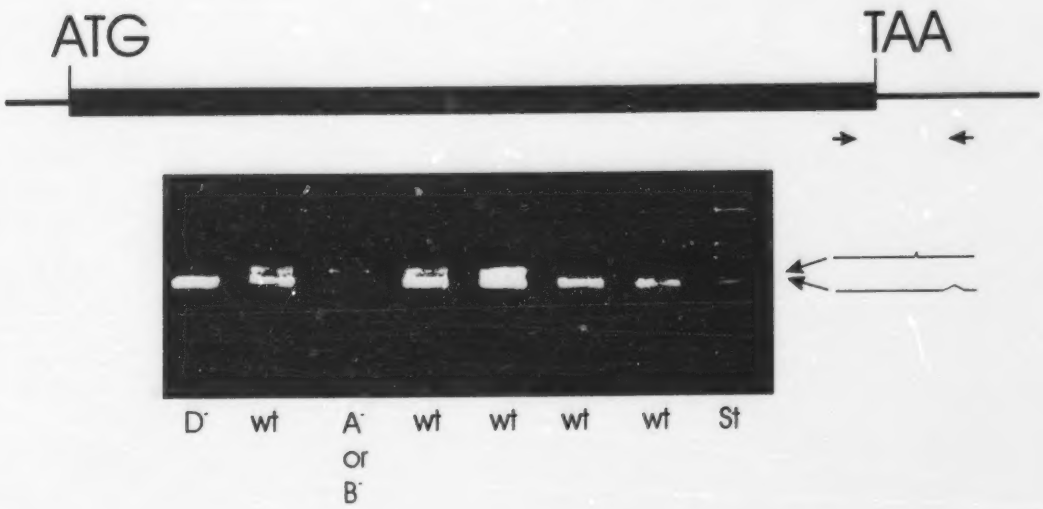

Figure 2: RT-PCR screening of wheat accessions for *Sbe2b* null allelles. Molecular weight standard in the rightmost lane is the 100 base pair ladder starting with 100 bp as the bottom band. D^- null D, wt, wild type, A^- or B^- : partial null A or B.

6.2 Identification of null SSII wheat lines:

A modified SDS-PAGE as described in the Materials and Methods section was developed to separate SSII from A, B and D genomes of wheat. Starch granule proteins were extracted from a few seeds in the initial screening, while from only embryo-less half seed portion of the progeny obtained from crossings. The corresponding embryo-portion of the seed was stored to be grown if necessary after SDS-PAGE and starch analysis. Fig. 1 shows a representative SDS-PAGE analysis of starch granule proteins and the separation

portion of the progeny obtained from crossings. The corresponding embryo-portion of the seed was stored to be grown if necessary after SDS-PAGE and starch analysis. Fig. 1 shows a representative SDS-PAGE analysis of starch granule proteins and the separation of SSII A, B and D genome products. The presence and / or absence of SSII was also confirmed by immunoblot analysis using SSII specific antibodies (Gao and Chibbar, 2000).

Screening of 505 wheat accessions resulted in two null A, three null B, two partial null A or null B SSII wheat lines (Table 1). We also found a wheat line with a partial D mutant allele, however, we were not able to find a complete null D wheat line. We further screened 192 Turkish wheat accessions and found three null D lines for the 100 – 115 kDa granule bound proteins. Two of these lines also exhibited mutation for granule bound starch synthase 1 (GBSS1, a key enzyme in amylose biosynthesis). Absence of any GBSS1 allele is undesirable since amylose production will be reduced. The seeds of the third line showed segregation for GBSS1, and only those seeds that have GBSS1 will be used from this line in a breeding program. Other wheat accessions that revealed null D allele were identified to be tetraploid wheat, and therefore, could not be used to develop triple null alleles in hexaploid wheat. However, null A and B alleles with partial D hexaploid wheat line were combined to investigate the effect on amylose production. Chinese wheat germplasm contains considerable variation for genes for starch biosynthtetic enzymes. For example the only known null D GBSS 1 line Bai Hua is of Chinese origin. Therefore, we analyzed 576 Chinese wheat accessions to look for a D genome null SSII wheat line. However, despite our extensive search, we have not been able to discover a null D SSII wheat line.

6.3 Stacking of Null SS I and II and/or Null SBEIIB Alleles

Double mutants were produced by crossing wheat lines with null SSII alleles for A (Turkey and Korea) and B (Turkey) genomes (Table 2). Eight individual plants with null SSII from A and B genomes were selected for introgression into a hexaploid wheat line HY417 and a durum wheat cultivar Sceptre. Thc progeny of these cultivars was analyzed by SDS-PAGE for null SSII alleles. Screening of the 72 F_2 half seeds from the HY417 cross has identified six double null lines (null A and B) (Table 3).

Crosses between the double mutants and the durum wheat cultivar Sceptre were performed to develop tetraploid wheat lacking the 100-115 kDa starch synthase. Screening of the 135 half-seeds (F_2 progeny) of the crosses identified no tetraploid null A and B lines, but 8 and 4 null A and D and B and D lines, respectively.

Table1: Wheat accessions analyzed for SSII mutations by SDS-polyacrylamide gel electrophoresis.

Origin	**Number of wheat accessions analyzed**	**Number of mutants for SSII encoded by the A, B and D genomes of wheat.**
Japan	79	
Australia	92	1 partial B mutant
China	136	1 partial A mutant 2 null B mutants 1 partial B mutant
Korea	12	1 null A mutant 1 partial A mutant
Turkey	181	1 null A mutant 1 null B mutant
USA	1	1 partial B mutant
Unknown	4	
Total	**505**	**9mutants**

Table 2: Wheat crossings made to produce SSII double null wheat lines.

SSII Alleles	**Origin**	**SSII Alleles**	**Origin**	**Resulting Progeny**
Null A	Turkey	Null B	Turkey	Null A+B (1)
Null A	Korea	Null B	Turkey	Null A+B (2)

Two wheat lines showed a null or partial null for starch branching enzyme. This approach will help us gain a better understanding of the effects of both starch synthases and branching enzymes on amylose production. Approximately 300 half-seeds from the

crosses with another identified wheat accession have been analyzed, and 33 null A and B alleles for the 110-115 kDa granule bound starch synthase were identified.

Table 3: Introgression of null A & B SSII alleles into wheat and durum backgrounds.

Pollen Parent	**Seed Parent**	**Seeds Analysed**	**Null A and B SSII**
Null A+B (1) or Null A+B(2)	HY417	192	13
	Sceptre	120	11 (null A & D) 7 (null B & D)
	SBEII null	562	51
	Partial SBEII null	192	17

Seven populations were developed to stack mutations for a 73Kda starch synthase (SS I). This starch synthase has also been suggested to have an important role in amylose synthesis. The wheat lines with *Ss1* null alleles had been reported in the literature and used for crossing experiments described in this section. The populations involved parents, which were spring and winter types and are generally not adapted to local growing conditions. The populations were advanced in bulk in 1998 and 1999 in order to eliminate late-maturing progeny. In 1999 single plant selections were made and the harvested seed was screened for kernel plumpness. In 2000, F_4 head rows (n=2,062) were late-seeded in order to select against progeny with a vernalization requirement. Approximately 500 lines were sampled at harvest. This material needs to be screened for the stacked mutations via two-dimensional gel electrophoresis. Once that has been done, further stacking with other mutants would be desirable to alter starch composition.

In addition to the above populations, another 20 populations with *Ss2* null A and B alleles were advanced in bulk (F_2 to F_4) in 2000.

6.4 Characterization of Wheat Lines Stacked for Null SSII and/or SBEIIb Alleles

The main objective of this project is to produce wheat lines with high amylose starch. Precise determination of amylose concentration is very difficult, because of the structure

hydrolysis releases glucose, which is quantitatively assayed spectrophotometrically. This method has variation in the range of three to four percent and it is also affected by the structure of glucan molecules. Therefore, we have previously reported the optimization of a high-performance liquid chromatography (HPLC) based method for amylose determination (Demeke et al., 1999). This method also takes into consideration the variation in the glucan polymer structure, reproducibility is very high and the variation in amylose concentration is less than a percentage point. In this method, starch is debranched using isoamylase and subsequently separated on a size exclusion column using high performance liquid chromatography. In this method amylose is excluded from the column earlier than the debranched amylopectin. The shape of the peak indicates the type of the molecules, while the integration of the area of amylose and amylopectin peaks indicates their concentration (Fig. 3).

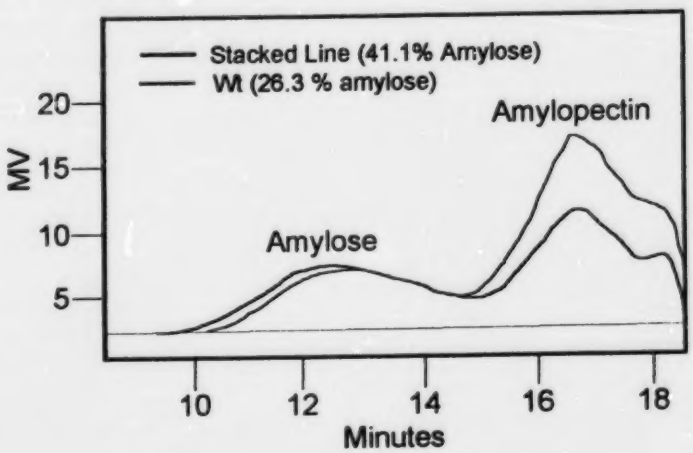

Figure 3: Amylose determination using the HPLC-SEC technique. Debranched starch from a wild type wheat and a wheat line stacked for null SSII and SBEIIb was separated as described in the methods section.

Progeny from crosses between the starch synthase II double mutants with a durum wheat (Sceptre, [F_4]) or the identified null D SBE or a partial SBE mutant [F_3] resulted in lines with amylose concentrations as high as 40%. Similar As an arbitrary guide, we selected

Progeny from crosses between the starch synthase II double mutants with a durum wheat (Sceptre, [F_4]) or the identified null D SBE or a partial SBE mutant [F_3] resulted in lines with amylose concentrations as high as 40%. Similar As an arbitrary guide, we selected 30% as an indication of high amylose concentration. The high amylose lines formed four to five percent of the progeny (Table 4). This is an interesting observation, which is at present difficult to explain. The number of genes involved and the triploid nature of wheat endosperm may contribute to the complexity of the system. Therefore, we need to follow this in subsequent generations to get a better understanding of the interaction of various genes in amylose synthesis and to develop stable high amylose wheat lines.

Table 4: Amylose concentrations in progeny of stacked null *Ss2* and *Sbe2b* alleles.

Cross	Seeds Analysed	High Amylose Seeds (> 30% Amylose)	Highest Amylose Concentration (%)
Sceptre//SSII null A&B alleles	573	32	41.4
Partial SBEIIb null// SSII null A&B alleles	189	8	41.1
SBEIIb null// SSII null A&B alleles	555	31	36.3

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Item (B) Deviations From Original Plan

As previously reported, we have focussed on both starch branching enzyme and 100-115 kDa starch synthases to identify single and double mutants and reconstitute single and double mutants of these gene alone and in combination to observe their effect on amylose production. We believe, that reduction in the starch synthase and starch branching enzyme activity will enhance our chances of developing high amylose wheat, and therefore, we have focussed our efforts on identifying and characterizing wheat germplasm with mutant alleles for these genes. Due to the focus of our research on two genes SsII and Sbe, we could not pursue the transgenic approach to increase amylose concentration.

ITEM "C".

ITEM "D". Equipment-